

Molecular cloning of the hepatitis A virus receptor from a simian cell line

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Using a eukaryotic expression system in combination with a monoclonal antibody (MAb) capable of blocking hepatitis A virus (HAV) adsorption, a cDNA clone was selected from a library of S.la/Ve-1 cells, a cell line that is highly susceptible to the virus. Sequence analysis of the cDNA revealed a single open reading frame that encoded a protein consisting of 460 amino acids. The deduced primary structure of the protein included a signal sequence, a transmembrane domain, four sites for N-linked glycosylation, cysteine residues attributable to an immunoglobulin domain and threonine clusters characteristic of mucin-like protein. By employing a vaccinia virus expression vector, the cDNA was expressed in HeLa cells where it induced marked HAV attachment which was specifically blocked by the MAb. The cDNA obtained was thus assumed to encode a functional receptor for HAV.

Hepatitis A virus (HAV) is the causative agent of hepatitis A, which infects via the faecal–oral route. The virus belongs to the family *Picornaviridae*, genus *Hepatovirus* and shares some physicochemical characteristics with members of the genus *Enterovirus*, but is distinct from these in its biological properties, showing slow growth in susceptible cells in culture and a lack of cytopathic effects (CPE) in infected cells (Lemon & Robertson, 1994). These unique biological properties of HAV have hampered precise and rapid assays of virus infectivity titres and have hindered analysis of the growth mechanisms of the virus at the molecular level, including identification of the cellular receptor, which is critical in understanding the pathogenicity of the virus. Previously, we have generated hybrid cell lines between marmoset liver and Vero cells that are highly susceptible not only to cell culture-adapted strains but also to wild strains of HAV (Ashida *et al.*, 1989). This prompted

us to attempt to identify the cellular receptor for HAV by employing one of the hybrid cell lines. Here we describe the isolation of cDNA encoding a membrane-associated glycoprotein which is a plausible candidate for the receptor. During the course of this study, Kaplan *et al.* (1996) reported similar results with an African green monkey kidney cell line (GL37), but there are several variations in the primary structure of these two cloned cDNAs.

To identify the HAV receptor, we used cells which were highly susceptible to the virus as immunogens to raise monoclonal antibodies (MAb) capable of blocking virus adsorption. A cDNA library was then made from the susceptible cells and cDNA from the library encoding an MAb-reactive product was selected. The cells used were S.la/Ve-1 cells, a hybrid between marmoset liver cells and Vero cells. The HAV strain used as the challenge virus was cell culture-adapted strain T.T. (Kojima *et al.*, 1981).

To prepare MAb against S.la/Ve-1 cells, the membrane fraction of 2×10^7 S.la/Ve-1 cells was mixed with an equal amount of complete Freund's adjuvant and used for immunization of BALB/c mice. At 6 weeks post-inoculation, mice received a booster injection with the same dose of immunogen mixed with incomplete Freund's adjuvant. Three days after the booster inoculation, spleen cells were fused with SP2/0-Ag14 myeloma cells (ICN Biomedicals) according to the conventional protocol (Oi & Herzenberg, 1980). When hybridoma colonies had grown, the culture supernatants were subjected to a two-step screening procedure to assess their ability to block virus adsorption. The first screening was to monitor virus growth inhibition by pretreatment of S.la/Ve-1 cells with the supernatants to block virus attachment. The hybridoma supernatants were added to 96-well microplate cultures of S.la/Ve-1 cells. Cultures were allowed to react at 4 °C for 30 min, washed with PBS, inoculated with HAV at an m.o.i. of 1 for 2 h to allow virus adsorption at 4 °C, washed twice and then incubated at 37 °C for 5 days to allow virus growth. At the end of the incubation period, culture wells were fixed with 80% methanol and assayed for HAV antigen by an enzyme immunoassay (EIA). Hybridoma supernatants showing significant inhibition of virus growth (inhibition of viral antigen development) were further tested directly for their ability to inhibit virus adsorption using labelled HAV. Cultures of S.la/Ve-1 cells in 24-well plastic microplates were washed

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-179
-120 GGAACACATTTTATGCTATTTTACAACTGAGGAAATAGAGTTTGAAGAGAACTCAGGAAGGCTCAGGGTATCCAGCATTTGTGAGTGACAGAGCTTGGATCTGAAACCGGGATCCCATTA
1 ATGCATCTCAAGTGGTCACTTAAAGCCTCATCTACATCTGGCAGATTTGTAGCCGATCTGTGATAAATGTTGATGGAGTGGCAGGTCTATCTATCACACTGCCTTCCGCTACAACGGA
1 M H L Q V V I L S L I L H L A D F V A D S V N V D G V A G L S I T L P C R Y N G
41 A I T S M C W N R G T C S V F S C P D G I V W T N G T H V T Y R K E T R Y K L L
241 GGAACCTTTACGCAGGATGTCTCTTGTACTATAGCAAAATACAGCTGTGCTGACAGTGGCATATATTTGCGGTGTTGACACAGTGGTGGTTCAATGACATGAAAATCACCATA
81 G N L S R R D V S L T I A N T A V S D S G I Y C C R V Q H S G W F N D M K I T I
361 TCATTGAAGATGGGCCACCCAGGGTCACTATTCCAATTGTGCAAACTGTTGCAAAAGCACCCTGTTCCAACGACAACGACAACGACCCCTTCCAACAACAACCCCTTCCAACGACA
121 S L K I G P P R V T I P I V R T V R T S T T V P T T T T T L P T T T L P T T
481 ACGACCTTCCAACGACAACGACCCCTTCCAACGACAACGACTCTTCCAATGACAACACCCCTTCCAACGACAACGACTGTTCCAACGACAACGACTCTTCCAACGACTCTTCCAACGACG
161 T T L P T T T L P T T T T L P M T T T L P T T T T T T T T T T T L P T T T L P T T T L P T T T L P T T T L P T T T L P T T T L P T T T L
601 ACTCTTCCAACGACTCTTCCAACGACAACGACTCTTCCAACGACTCTTCCAACGACAACGACTCTTCCAACGACAACGACTCTTCCAACGACAACGACTCTTCCAACGACAACGACTCTG
201 T L P T T L P T T T T L P T T T L P T T T T L P T T T M T L P M T T T T L P T T M T T L
721 CCAACGACGACGACCCCTTCCAACGACGACTCTGCAACGATGACTCTTCCAACGACAACGACTCTTCCAACGACAACGACTCTGCAACGACAACGACTCTGCAACGACAACGACTCTT
241 P T T T T L P T T T T M V S T T F V P F T P L P M Q N H E P V A T S F S S P Q P A A E
841 CCAACAACAACAACCTGCAACGACAACCATGGTTTCTACCTTTGTTCTTCCAACCATGCGCCATGCGAAGCAGTACCCTTCCACTCTTCCACTCAGCCAGCAGAA
281 P T T T T L P T T T T M V S T T F V P F T P L P M Q N H E P V A T S F S S P Q P A A E
961 ACCACCTGTGACCTGGGAGCAACAAGGACACAACCCACGCTCACCATTGTACTCTTACAACAGATGGGAGTGACACCGTGACAGAGCTTCCAGATGACAGAGCTTGGAAATA
321 T H P V T L L G A T R T Q P T S S P L Y S Y T T D G S D T V T E S S D G L W N N
1081 AATCAAATCAATGTCCCGAAGCATAGTCCACAGATGGTCAACACCCTGAAAGGATCTATGCTGGAGTCTGTATTTCTGTCTTGGTGTCTTGTCTGTTTTGGTGTCTGCTCATTGCC
361 N Q T Q L S P E H S P Q M V N T T E G I Y A G V C I S V L V L L A V L G V V I A
1201 AAAAAGTATTTCTTCAAAAAGGAGATTCAACAACAAAGTGTTCATTTAGCAACCATCAATTTAAAACCTTTGCAAAATGCAGTTAAAAGGAAGTCCACGCAAGAACAATATCTACATT
401 K K Y F F K K E I Q Q L S V S F S N H Q F K T L Q N A V K K E V H A E D N I Y I
1321 GAGAATAATCTTTATGCCATGAACCAAGCCAGTGGTGTCTTTGAGAGTTTACGCCAATGACTGCGAAGACTGAACATATCGGACGCTTTTGAGACTCCAAGACAATTTTTCTGTTT
441 E N N L Y A M N Q D P Q M V N F T E S L R P *
1441 CAGTTCATCTGGCCCTTCCAACATGTCAGTGCACCTGGGTAGAGTAACTCTTCTGCTCCAACCTGTGTATAGTCAACCTCATCATTAATGTAGTCTAATTTTTTGTGCTAAAACAGGTT
1561 CAATCAATCCCTCGATCTGTAGAGTCTCTGTCAACATGAACACTTGAAGATTTGATGTTCTCTTTAGACCCATATAATCTGTATCCATCAGAGAGAATAGTCACGGAAACATA
1681 GCAAAATGAACTTCTGCTTGGCCATCAAAGCTGTGCAAGAGGGGAAATCTGTCTTAAAAATCAGCAATCCGATGTGAGACTTCAATTTGAAAGCATATCATGATAATCTCTGTCTTCTA
1801 TATGATACTTCAAAATGTCATTCTTCTGTGTTTCCAAGGTTTCAAATCATGGGTTTAAATGCTCCATGGGGAATAAAGTGAGTTAAATCCACCTAAAAAATAAAAAA
1921 AAAAAAATAAAAAA

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Fig. 1. Primary structure of the cDNA encoding the putative HAV receptor. The recombinant plasmid (pCDM8-2H4) was selected as described in the text. The cDNA insert was recovered as an *Xba*I-*Hind*III fragment and subcloned into pBluescriptII for construction of nested deletion sets using an ExoIII/Mung bean deletion kit (Stratagene). The sets obtained were subjected to nucleotide sequence determination employing *Bca*BEST Dideoxy Sequencing kits (Takara, Japan). To analyse the nucleotide sequences, GENETYX-Σ (Software Development Co., Japan) was used. Functional regions/sites in the primary structure are indicated as follows: boxed aa 1-21, signal sequence; boxed aa 379-400, transmembrane domain; boxed C residues, cysteine residues involved in the putative immunoglobulin domain (Altschul *et al.*, 1990; Thompson *et al.*, 1994); and underlined amino acid triplets, N-linked glycosylation sites.

twice with PBS and reacted with hybridoma supernatants at 4 °C for 30 min; the hybridoma supernatants were removed and cultures were inoculated with ¹²⁵I-labelled HAV (5000 c.p.m. per well) at 4 °C for 2 h. HAV used as the inoculum was sucrose density gradient-purified, radiolabelled, column-sieved, density gradient-purified again to remove free radioisotopes and resuspended in Dulbecco's modified MEM (Zajac *et al.*, 1991). Cultures were washed with PBS three times and solubilized with 1% SDS. Cell lysates thus obtained were subjected to gamma counting to detect cell-associated radioactivities in each well in order to assess the reduction in HAV adsorption caused by pretreatment of cells with the supernatants. After these screening procedures, a hybridoma cell line was obtained which was a stable producer of MAb 2H4, a MAb that blocked HAV adsorption. The MAb was of the IgG2b isotype.

cDNA from S.la/Ve-1 cells was prepared according to the method of Gubler & Hoffman (1983). A *Bst*XI-*Eco*RI adapter (Invitrogen) was ligated to the cDNA which was then inserted into the expression vector pCDM8 (Aruffo & Seed, 1987) at the *Bst*XI site, and then transfected into COP-5 cells (Tyndall *et al.*, 1981) in 6 cm plastic tissue-culture dishes using DEAE-dextran (Sigma). The panning method was used for selection of cDNA in the eukaryotic expression system (Seed & Aruffo, 1987). COP-5 cells (4 × 10⁷), 48 h after transfection with the cDNA, were dispersed in 0.02% EDTA and resuspended in

2 ml of reaction medium (PBS supplemented with 0.02% EDTA and 5% FBS) containing 20 μg of MAb 2H4 and reacted at 0 °C for 30 min. After the reaction, cells were washed by centrifugation at 1500 r.p.m. for 4 min, resuspended in 4 ml 0.02% EDTA and plated into ten 6 cm panning Petri-dishes for immunofixation for 4 h at room temperature. The panning dishes were coated with anti-mouse IgG goat Igs (Rockland) and each contained 3 ml of reaction medium. Dishes were washed ten times with the reaction medium to remove unfixed cells and supplemented with 400 μl per dish of 0.6% SDS containing 10 mM EDTA. Finally, the resultant cell lysates were extracted using the Hirt method (Hirt, 1967). The pool of recombinant plasmids thus obtained was further enriched for the working components by another three cycles of panning selection, where the unit cycle consisted of propagation of the plasmids by MC1061/P3 (a bacterial host), transfection of COP-5 cells with the propagated plasmids for expression/immune selection and Hirt recovery of the plasmids selected. After this enrichment, the final samples were subjected to specific cloning of the working plasmids with the following protocol: transformation into MC1061/P3, extraction of plasmid DNA, transfection of COP-5 cells with the cloned molecules for expression and selection by EIA. Eventually, two out of a total of 96 recombinant plasmids clones were found which encoded proteins reactive with MAb 2H4. These two plasmids gave cDNA inserts of identical size (2.1 kb); one

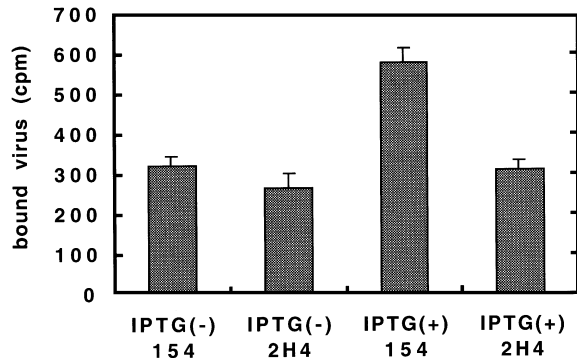


Fig. 2. Induction of HAV receptor activity in HeLa cells and blocking of activity by MAb 2H4. HeLa cell cultures were inoculated with the recombinant vaccinia virus containing the cDNA insert, and incubated for 18 h in the presence of IPTG to induce HAV receptor activity. After cultivation, cells were pretreated with MAb 2H4, then infected with ^{125}I -HAV to assay for cell-associated virus in comparison with controls. Bars indicate average c.p.m. values of triplicate samples with standard errors. IPTG (-)/(+), absence/presence of IPTG for induction of HAV receptor activity; 154, control MAb with activity different from that of MAb 2H4; and 2H4, MAb to HAV receptor. For details of the genomic construct of the vaccinia virus expression vector and its use, see Rodriguez & Smith (1990). For details of the cell-associated virus assay using ^{125}I -HAV, see text.

(pCDM8-2H4) was arbitrarily selected as encoding the HAV receptor and used for further analysis.

The sequence of the cDNA insert was determined by the Sanger method (Sanger *et al.*, 1977) and is shown in Fig. 1. The total span was 2080 nt (nt -179 to 1901) excluding the poly(A)⁺ tail and included a single open reading frame (ORF) located from nt 1 to 1380 encoding a polypeptide of 460 amino acids. Upstream of the polypeptide, there was a leader signal peptide (aa 1 to 21, boxed) followed by the trunk structure which spanned aa 22 to 460, in which a transmembrane domain (aa 379 to 400, boxed) and four putative *N*-linked glycosylation sites (underlined) were observed. Cysteine residues were detected at aa residues 36, 46, 52, 57, 104, 105 and 385; those at positions 36 and 105 might yield an immunoglobulin domain (Williams & Barclay, 1988) resembling that found in the poliovirus receptor (Mendelsohn *et al.*, 1989). An additional feature of interest was the mucin-like protein component in the region from aa 140 to 290, where threonine residue cluster repeats with the sequence 'TTTTLP' were observed (Toribara *et al.*, 1991; Strous & Dekker, 1992). The primary structure of this polypeptide was compared with that of the HAV receptor reported by Kaplan *et al.* (1996); the two proteins differed by eleven insertions, two deletions and seven residue replacements, and were 460 aa and 451 aa, respectively, although the principal constructs were practically identical (95.7% at the amino acid sequence level).

To confirm that the cDNA encoded an HAV receptor polypeptide, we attempted to examine the receptor activity of the cDNA product using other conventional vectors (pMAMneo, pcDNA3), but unsatisfactory results were obtained. So, we used the vaccinia virus expression system

with HeLa cells, which are negative for HAV adsorption, in a preliminary experiment. The cDNA insert in the pCDM8-2H4 plasmid was transferred to the recombination vector pPR35 (Rodriguez & Smith, 1990) and recombinant vaccinia viruses were produced by homologous recombination (Mackett *et al.*, 1984). The vector has inducible promoter elements enabling expression of exogenous DNA on induction with IPTG (Wako). HeLa cells in 24-well plates were inoculated with the recombinant virus at an m.o.i. of 5 with 5 mM IPTG for expression of the cDNA. At 18 h, cells were pretreated with MAb 2H4 for 30 min at 4 °C, then inoculated with ^{125}I -HAV to allow detection of receptor activity and its specific inhibition by the MAb. As shown in Fig. 2, in the absence of IPTG [IPTG(-)] virus adsorption remained at a low level with a value of around 300 c.p.m. for cell-associated virus. Virus adsorption under these conditions was not influenced by pretreatment of the cells with MAb 2H4 [IPTG(-), 2H4]. After induction of cDNA expression, however, a significant increase in virus adsorption was observed with a value of nearly 600 c.p.m. [IPTG(+), 154 (control MAb with a different activity to that of 2H4)]. This increase in virus adsorption was completely abolished when the cells were pretreated with MAb 2H4 [IPTG(+), 2H4], reducing the value to the level observed in the non-induced state. Thus, this experiment clearly showed that the product of the cloned cDNA was capable of acting as an HAV receptor for virus adsorption and that MAb 2H4 specifically blocked binding to the receptor.

For further examination of the HAV receptor blocked by MAb 2H4, the MAb was employed to detect the receptor in the original cells used in experiments. S.la/Ve-1 cells were metabolically labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine, and the lysates were subjected to immunoprecipitation by MAb 2H4, followed by SDS-PAGE (Fig. 3). A single broad band with a mobility in the range of 95–120 kDa was obtained from S.la/Ve-1 cells (lane 1); no band was obtained with the negative control, A31 cells (lane 2), a murine cell line lacking HAV susceptibility. The broad mobility of the band observed and the discrepancy between the molecular size (95–120 kDa) indicated by electrophoresis and that (50 kDa) deduced from the ORF, suggests that the polypeptide is a highly glycosylated glycoprotein.

In the present study, MAb 2H4 raised against S.la/Ve-1 cells, a cell line highly susceptible to HAV, blocked HAV adsorption; using this MAb a cDNA clone was selected from the cell line in a eukaryotic expression system, and the cDNA was shown to encode a product which allowed HAV adsorption. That this cDNA encoded a HAV receptor was also supported by a separate observation using MAb 2H4 for immunofluorescent staining of cells. The MAb stained HAV-susceptible cell lines such as GL37 (Kaplan *et al.*, 1996), Vero (Locarnini *et al.*, 1981), BSC-1 (Binn *et al.*, 1984) and LLC-MK2 (Provost, 1984), all of which have been shown to support virus growth efficiently, whereas cell lines known to be refractory to HAV infection, such as HeLa, KB, L and A31, were not stained

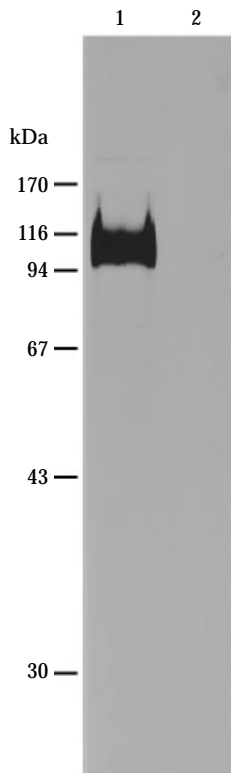


Fig. 3. Recovery of polypeptide reactive with MAb 2H4 from S.la/Ve-1 cells by immunoprecipitation. S.la/Ve-1 cells in 6 cm plastic tissue-culture dishes were metabolically labelled with a mixture of [³⁵S]methionine and [³⁵S]cysteine (100 μ Ci per 1.5 ml of dish; EXPRE³⁵S³⁵S Protein Labelling Mix, NEN) for 4 h. Labelled cells were washed twice with PBS and lysed in 400 μ l of RIPA buffer (10 mM Tris-HCl pH 7.4, 1% NP40, 0.1% SDS, 0.1% sodium desoxycholate, 150 mM NaCl, 1 mM EDTA and 10 μ g/ml of aprotinin). The cell lysate was clarified by centrifugation at 10 000 *g* for 20 min and the supernatant was supplemented with 30 μ l of 50% (v/v) Protein A-Sepharose (Pharmacia) for non-specific adsorption of labelled polypeptides at 4 °C overnight. After adsorption, the resin was removed by centrifugation and supernatants were supplemented with 5 μ g of MAb 2H4 and reacted for 1 h at 4 °C, followed by another addition of 30 μ l of Protein A-Sepharose for specific adsorption of the Ag-Ab complex. After incubation at 4 °C for 1 h, the resin was washed five times with PBS; the bound polypeptide was eluted into 30 μ l of electrophoresis sample buffer and analysed by SDS-PAGE (12.5% gel) and autoradiography. Lane 1, S.la/Ve-1 cells; lane 2, A31 cells, a murine cell line refractory to HAV infection. The sizes (kDa) of the protein markers are shown to the left of the gel.

at all (data not shown). Although these results did not conclusively demonstrate that the product of the cDNA obtained is the functional receptor required for growth of HAV, it seems worthwhile to further analyse its biochemistry and biology. Such studies will clarify the functional authenticity of the product, which would ultimately facilitate elucidation of the virus growth mechanism and pathogenicity.

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